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Monitoring of Environmental Cancer Initiators through Hemoglobin Adducts by a Modified Edman Degradation Method

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Tissue doses of cancer initiators/mutagens are suitably monitored through hemoglobin adducts formed *in vivo*, but the use of this method has been hampered by a lack of sufficiently simple and fast procedures. It was previously observed that when the N-terminal amino acid in hemoglobin, valine, is alkylated it is cleaved off by the Edman sequencing reagent, phenyl isothiocyanate, in the neutral-alkaline coupling medium, as opposed to the acidic medium required by normal amino acids. Based on this principle, conditions for a functioning procedure for gas chromatography/mass spectrometry (GC/MS) determination of N-terminal alkylvalines in hemoglobin were worked out. Derivatizing the protein in formamide solution with pentafluorophenyl isothiocyanate, using a ^{2}H-alkylated protein as internal standard, and applying on-column injection during analysis, permit reproducible determination of hydroxyethylvaline and other adducts down into the dose range where cancer risks may be considered acceptably low.

KEY WORDS: hemoglobin adducts; hydroxyethylation; ethylene oxide; pentafluorophenylthiohydantoins; N-terminal alkylvaline; gas chromatography/mass spectrometry.

Epidemiological studies of tumor diseases indicate that possibly 90% of current incidences (1) may be due to environmental factors, environment being taken in a very broad sense (2). Although considerable knowledge has been gained we still lack more precise information on causative agents, especially the cancer initiators. A main reason for this is the limited ability of present experimental and epidemiological methods to detect risk factors (3). To overcome these limitations, improvements of methods are required in several respects [cf. Refs. (4–6)], especially (a) increase of statistical power ("sensitivity"), (b) early detection of risk, (c) specificity to, i.e., ability to identify, causative agents, even a priori unknown ones, in complex mixtures, and (d) general usefulness for risk estimation.

Most if not all genotoxic chemical agents are, or are metabolized to, electrophilic reagents with an ability to react with DNA, RNA, and proteins in tissues (7). The formation of adducts of ultimate cancer initiators and mutagens with macromolecules offers possibilities to utilize the high power of chemical analysis in testing and monitoring methods (8–10). Hemoglobin has been found suitable for this purpose because it is accessible in large amounts in blood samples and the determination of hemoglobin adducts has advantages compared to determination of DNA adducts (11–13). The use of conversion of adducts to certain amino acids in hemoglobin as the observed endpoint appears able to fulfill the above requirements (a)–(d). This method permits, in principle, monitoring of tissue doses of most known genotoxic chemicals.

The quantitation of alkylated amino acids, primarily histidine, in hemoglobin has so far been based on tedious work-up of samples [total hydrolysis of protein, ion-exchange separations of fractions containing the species to be determined (13,14)] which has hampered

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a general application of hemoglobin dosimetry. This was a main reason for initiating the present study of improvements of the method.

Jensen *et al.* (6) showed that alkylated N-terminal valine in hemoglobin treated with radioactively labeled ethylene oxide was specifically split off when Edman degradation (15) was applied to the protein:[1] In this way the alkylated valine could be separated from the rest of the protein to which the unalkylated valine is still bound. It was suggested that a development of a modified degradation procedure, based on this observation, could lead to a rapid method for determination of hemoglobin adducts. To achieve the required sensitivity, the conventional Edman reagent, phenyl isothiocyanate, was replaced by pentafluorophenyl isothiocyanate (16,17).

Although synthesized standard pentafluorophenylthiohydantoins of *N*-alkylvalines (alkylvaline PFPTHs)[2] offered high analytical response, several problems remained to be solved. The difficulties involved may be illustrated by mentioning that the determination and exact quantitation of one chemically changed amino acid out of 10^5–10^7 normal ones was aimed at (this corresponds to ca. 1–0.01 nmol adduct per gram hemoglobin; see further discussion of sensitivity under Discussion). Already in the normal Edman polypeptide sequencing, i.e., determining the natural amino acids, difficulties appear due to impurities, by-products, and decay of the derivative to be determined, though in that case a major part of by-products are eliminated by extraction (18). In the present modified procedure complications arise from the fact that impurities and by-products are coextracted with the alkylvaline PFPTHs which, in the case of hydroxyethylated N-terminal valines, have prevented the detection of less than 1 nmol adduct per gram hemoglobin (17). Considering these problems the development of a highly sensitive method for determining alkylated amino acids, which could be used for risk assessment in complex exposure situations and which should be fast and simple, represented no doubt a challenge.

This paper reports on development of a functioning method for the determination of alkylvalines in hemoglobin in the above-mentioned range of levels. This is illustrated by observed alkylation levels (hydroxyethylations) in hemoglobin from humans and rodents without known exposure. Furthermore, the reproducibility and sensitivity of the method are demonstrated.

MATERIALS

Pentafluorophenyl isothiocyanate (purum) was obtained from Fluka, Buchs, Switzerland, and was used without further purification. Ethylene oxide was obtained from Fluka; [^{14}C]ethylene oxide (22.0 mCi/mmol) was from Amersham International Ltd., Amersham, Buckinghamshire, England; and [$^2H_4$]-ethylene oxide and [$^2H_3$]methyl iodide were from Merck Sharp & Dohme Canada Ltd., Montreal. Propylene oxide was obtained from Merck–Schuchardt, Darmstadt, Federal Republic of Germany. The styrene oxide (pure, from Koch–Light Laboratories Ltd., Coinbrook, Buckinghamshire, England) was distilled prior to use. [1-^{3}H]Ethan-1-ol-2-amine hydrochloride (8.8 Ci/mmol) was obtained from Amersham International Ltd. 2-Bromoisovaleric acid, analytical grade, was obtained from Fluka. Methylvaline was available from Sigma, St. Louis, Missouri. All other chemicals and solvents were of analytical grade. *The pentafluorophenyl isothiocyanate is to our knowledge not evaluated toxicologically, but in view of its volatility and high reactivity it certainly presents a health hazard which should be given due consideration when it is used in laboratory work.*

[1] A cleaving-off of methylated N-terminal amino acids in alkaline coupling solution has already been described by Chang (40) as an explanation of the overlapping with the succeeding amino acid which is sometimes observed in Edman sequence determination.

[2] This simplified nomenclature is used to indicate the amino acid from which the thiohydantoin is formed. A proper name of alkylvaline PFPTH would be 1-alkyl-L-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin.

METHODS

(A) Synthesis of alkylated valines. *N*-Methyl-DL-valine was commercially available. *N*-(2-Hydroxyethyl)-DL-valine and *N*-(2-hydroxypropyl)-DL-valine were synthesized by reacting *RS*-2-bromoisovaleric acid with the respective alkylamine (19). *N*-(2-Hydroxy-2-phenylethyl)-L-valine, prepared from L-valine and styrene oxide, was a gift from Dr. C. J. Calleman, Stockholm. *N*-(2-Hydroxy-[^{3}H]ethyl)-DL-valine (sp act 8.8 Ci/mmol) was synthesized from [^{3}H]ethanolamine and *RS*-2-bromoisovaleric acid with a modification of the method described by Calleman (19).

(B) Alkylation of blood in vitro. A solution of the alkylating agent ([$^2H_3$]methyl iodide, styrene oxide, propylene oxide, [$^2H_4$]ethylene oxide, ^{14}C- or unlabeled ethylene oxide) was added to whole blood or erythrocytes (10^1–10^2 μmol/g hemoglobin or ca. 10 μCi/g hemoglobin) and the blood was incubated at 37°C for 2 h. The reaction mixtures were allowed to stand at room temperature overnight. Globin was prepared as described below. (It is preferable to alkylate whole blood in cases where an alkylating pattern identical with the pattern obtained *in vivo* is desired.)

(C) Isolation of globin from blood. The blood was suspended in 0.9% sodium chloride containing heparin, and plasma and erythrocytes were separated at 500*g*. The erythrocytes were washed with 0.9% sodium chloride (3 times) and then lysed by adding 1 vol of water. The cell membranes and debris were sedimented at 20,000*g*. The supernatant was dialyzed overnight against 0.001 M phosphate buffer, pH 7. Globin was precipitated by ethyl acetate from an acidified solution in 2-propanol/water (6:1), washed, and dried as described by Mowrer *et al.* (17). In some cases globin samples used in the present study were precipitated directly after lysing the red cells.

(D) Quantitative determination of hydroxyethylation in globins alkylated in vitro. Globin alkylated with ethylene oxide or [$^2H_4$]ethylene oxide with addition of globin alkylated with high-specific-activity ^{14}C-labeled ethylene oxide was hydrolyzed and separated by ion-exchange chromatography using a Dowex 50W-X4 resin [for procedures see Ref. (20)]. The hydroxyethylvaline fraction, traced by the added radioactivity, was determined on an amino acid analyzer. The levels of labeled and unlabeled hydroxyethylvaline in the globin samples were quantified; as for the [^{2}H]hydroxyethylated globin, see Farmer *et al.* (41). It was found that hydroxyethylvaline accounted for 11% of the total radioactivity in the [^{14}C]hydroxyethylated globin, other major products being hydroxyethyl-cysteine and -histidines.

(E) Derivatization of valine and N-alkylvalines. Two milligrams of the respective valine (cf. Table 1) was dissolved in a mixture of 1 ml of 0.5 M potassium hydrogen carbonate and 0.5 ml 1-propanol (pH meter recording 8.6). A 5-μl amount of pentafluorophenyl isothiocyanate was added. The reaction mixture was warmed at 45°C for 2 h (1 h in the case of unsubstituted valine) in a shaking water bath. The reaction mixture was extracted with *n*-heptane (2 + 2 ml) in the case of valine to eliminate by-products and in the case of alkylated valines to isolate the desired product, the respective alkylvaline PFPTH. The aqueous solution of the derivative of unsubstituted valine was then acidified with 0.5 ml of 6 M HCl, and *N*-(pentafluorophenylthiocarbamoyl)valine (PFPTC-valine) started to precipitate. 1-Propanol (0.5 ml) was added to dissolve the precipitate and the solution was heated at 80°C under occasional shaking for 0.5 h. The valine pentafluorophenylthiohydantoin (Val-PFPTH)[3] was then extracted with *n*-heptane (2 + 2 ml). The heptane–propanol extract of the valine or alkylvaline PFPTH was evaporated under nitrogen at 60°C and then redissolved in 1 ml toluene and washed with 2 ml water. A solution suitable for GC/MS determination was prepared by dilution in toluene. The yield was determined to 90–95% with ^{3}H-labeled HOEtVal. It should be noted that due to the difference in pK_a of

[3] Compare footnote 2.

TABLE 1

STUDIED VALINES, THEIR PENTAFLUOROPHENYL ISOTHIOCYANATE DERIVATIVES (PFPTHs), AND SUGGESTED ORIGIN OF ALKYLVALINES IN HUMAN HEMOGLOBIN

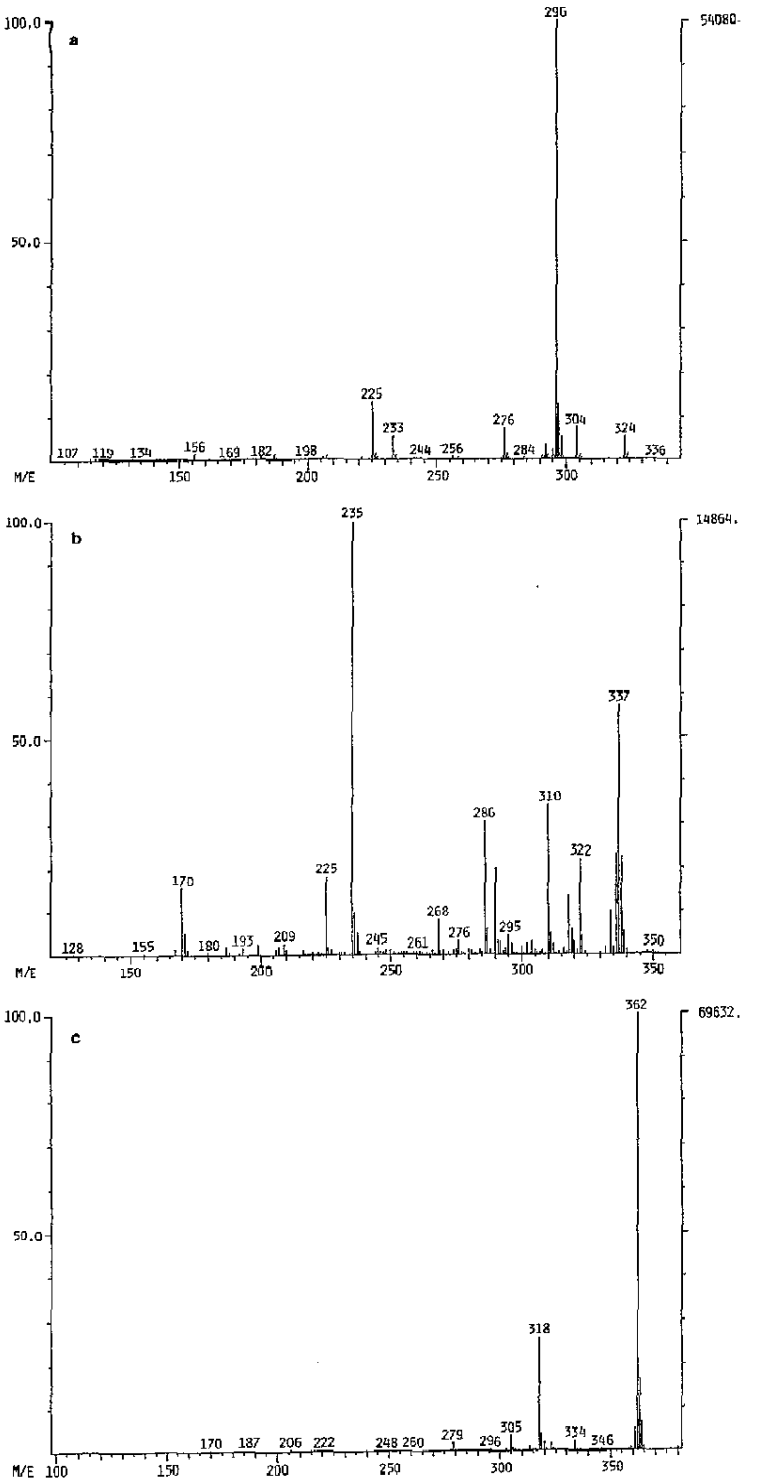
Amino acid	PFPTH	Alkylating agent
Valine	Val-PFPTH	
Methylvaline	MeVal-PFPTH	Endogenous (21) and exogenous methylating agents
2-Hydroxyethylvaline	HOEtVal-PFPTH	Exposure to i.a. ethylene oxide, ethene [also formed endogenously, cf. Ref. (22,23)], 1,2-dichloroethane
2-Hydroxypropylvaline	HOPrVal-PFPTH	Exposure to i.a. propylene oxide, propene
2-Hydroxy-2-phenylethylvaline ("dihydrohydroxystyrylvaline")	HOStVal-PFPTH	Exposure to styrene oxide, styrene

the amino group in peptide-bound and free valines, derivatization of free valines at pH lower than 7 as used for derivatization of globin will give low yields.

(F) Derivatization of globin samples. A 50-mg amount of globin was dissolved in 1.5 ml formamide (pH meter recording 5.3) and a suitable amount of internal standard (globin alkylated with [$^2H_4$]ethylene oxide) was added (cf. Table 2). Pentafluorophenyl isothiocyanate (7 μl) was added. For various practical reasons the initiation of the derivatization reaction was carried out slowly at room temperature overnight with tilting of the tube back and forth. To increase the yield the reaction was completed in a shaking water bath at 45°C for 1.5 h before extraction with ether (2 + 2 + 1 ml). (The pH meter recorded a drop of 0.5 pH units during reaction.) The ether extract was evaporated under nitrogen. After dissolution in 1 ml toluene the sample was washed under tilting twice with water (2 + 2 ml), twice with freshly prepared 0.1 M disodium carbonate (3 + 3 ml) for 3–5 min (to hydrolyze by-products), and then again with water (2 ml). After each washing the phases were separated by centrifugation at 500*g*. The toluene and volatile hydrolyzed by-products were carefully evaporated under nitrogen at 60°C. The sample was finally redissolved in 50 μl toluene for analysis. The yield of HOEtVal-PFPTH in this derivatization reaction was 35–40%, which was demonstrated by derivatization of [^{14}C]ethylene oxide-treated globin ([^{14}C]HOEtVal independently quantified in hydrolyzed globin as described in paragraph D above).

(G) GC/MS analysis. The samples were analyzed on a Finnigan 4021 GC/MS instrument in the negative ion chemical ionization mode. The operating parameters (17) for the mass spectrometer were as follows: methane reagent gas at an ion source pressure of 30–40 Pa (0.25 to 0.30 Torr); ion source temperature 200°C; filament operated at 16–25 eV. The operating parameters for the gas chromatograph were: helium carrier gas at constant pressure (1.4×10^5 Pa); temperature programming 10°C/min from 100 to 270°C. Two types of crosslinked quartz capillary columns were tested, SE-54 (1% vinyl, 5% phenyl methyl silicone) and OV-1701 (7% phenyl, 7% cyanopropyl methyl silicone). A 25-m OV-1701 (0.2 mm i.d., 0.5-μm phase thickness)

FIG. 1. Mass spectrum (negative ions) of different valine PFPTHs. (a) Valine PFPTH. (b) Methylvaline PFPTH. (c) Hydroxypropylvaline PFPTH.



indicated a good reproducibility and a high sensitivity and was therefore selected for the determination of low levels of alkylvaline PFPTHs. One microliter of the toluene solution of PFPTHs was injected directly (on-column injector OCI-3, Scientific Glass Engineering, Ltd., Australia). Initial tests were made with the split/splitless injector supplied with the instrument.

RESULTS

Procedure for the determination of N-terminal valines in hemoglobin. Although the need for further optimization work is indicated, the present work has led to a functioning procedure as given under Methods (paragraphs C, F, G) for the determination of low levels of alkylvalines formed in reactions of hemoglobin with low-molecular-weight alkylating agents.

GC/MS analysis of standard PFPTHs. The negative ion mode which was used for analysis is particularly convenient for the PFPTH derivatives of alkylated valines [cf. Ref. (17)]. Negative ion spectra of some of the examined compounds are presented in Fig. 1a–c. The 2-hydroxyalkylated substances yield only two major ions, both useful for analytical work, one at M − 20 amu, due to loss of HF from the parent molecule, and the other at 318 amu, due to loss of HF and RCHO (R = H, CH_3, etc.). Correspondingly, the [$^2H_4$]-hydroxyethylvaline standard gives mass fragments at 352 amu (−HF) and 320 (additional loss of C^2H_2O). Valine PFPTH and methylvaline PFPTH exhibit more complicated decay patterns. They lose a hydrogen or HF to give fragments at M − 1 and M − 20 amu, respectively; other fragments are not yet interpreted. The two most intense ions for each substance were selected and used when operating the instrument in the multiple ion detection (MID) mode. A possibility remains that rearrangement of a precursor to PFPTH—see Mechanism under Discussion and Refs. (15a,24)—occurs in the mass spectrometer.

HOEtVal-PFPTH (M_m 368) and its deuterated analog (M_m 372) used as internal standard were not resolved by the capillary column. However, the mass fragmentogram (see Fig. 2) reveals that the [$^2H_4$]HOEtVal-PFPTH elutes just ahead of the HOEtVal-PFPTH. HOPrVal-PFPTH and HOStVal-PFPTH give rise on certain columns (e.g., SE-54) to two peaks of approximately equal intensity [cf. Ref. (17)], which probably represent diastereomeric forms due to reactions of racemic propylene oxide and styrene oxide, respectively, with L-valine [cf. Ref. (25)]. Such formation of diastereomers would take place also if the L-amino acid is racemized during some step of the reaction (26). Whereas propylene oxide is expected to be substituted on carbon-1, both oxirane carbons are expected to react in the case of styrene oxide (27) with the formation of an additional pair of diastereomers in consequence.

Figure 3 illustrates the determination of the hemoglobin adducts investigated in the present study: PFPTHs of methyl-, 2-hydroxyethyl-, 2-hydroxypropyl-, and 2-hydroxy-2-phenylethyl-valines in globin treated *in vitro* with [$^2H_3$]methyl iodide, ethylene oxide, propylene oxide, and styrene oxide.

Reproducibility and linearity. Initial tests with HOEtVal-PFPTH indicated nonlinearity and poor reproducibility in the GC/MS analysis [variation up to a factor 2 in ion intensities between replicate injections; cf. Ref. (17)].

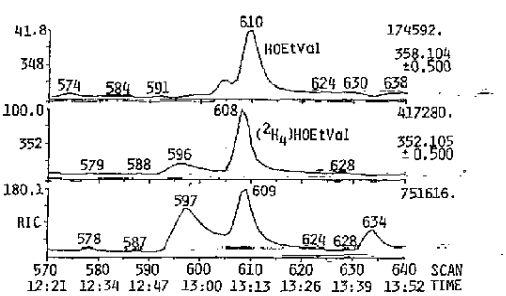


FIG. 2. Mass chromatogram (negative ions, MID 6 ions) from globin derivatized with pentafluorophenyl isothiocyanate showing the background hydroxyethylation (0.40 nmol HOEtVal/g globin) in a person without known exposure.

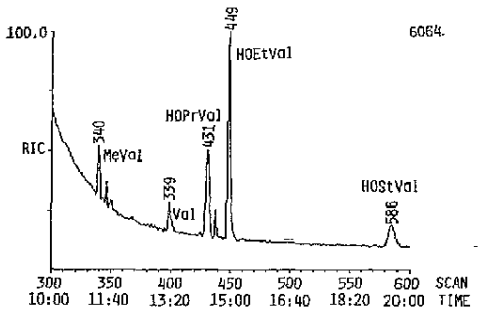


FIG. 3. Mass chromatogram (negative ions, full scan) of PFPTHs from globin treated *in vitro* with different alkylating agents to a content of approximately 100 nmol/g globin of each adduct (the total content of N-terminal valines is 62,500 nmol/g globin).

Decomposition of the PFPTH in the split/splitless injector was suspected to be a cause of this variation [cf. Ref. (18)]. The first step toward sufficient reproducibility was taken by installation of an on-column injector. This led to quite reproducible results with standard PFPTHs (less than 5% variation in peak areas for replicate injections) and was linear at least over the range 0.1–10 pmol.

The reproducibility was also found to be affected by the purity of the derivatized globin sample (necessity of minimizing disturbing by-products, see Discussion below).

The addition of a ^{2}H-alkylated globin standard before derivatization created independence of yield and of response linearity and therefore gave improved reproducibility. Quantification was based on peak areas.

To check the linearity and reproducibility of the method under the optimized conditions, analyses were carried out on globin samples, with and without addition of globin treated with ethylene oxide, and with different amounts of the internal standard, [$^2H_4$]-ethylene oxide-treated globin. In such mixtures HOEtVal was determined from the ratio HOEtVal/[$^2H_4$]HOEtVal as shown in Table 2. The table shows that the untreated globin studied in this case has a background level of 0.23 nmol HOEtVal/g globin and that up to 36 nmol HOEtVal/g is determined correctly, independent of the amount of internal standard added. The highest level of HOEtVal (180 nmol determined in the presence of 1 nmol/g Hb deuterated analog) gives too low a value probably due to saturation effects in the GC/MS analysis. To avoid these effects it is desirable not to exceed a factor of 30 in either direction in the ratios of determined to standard

TABLE 2

DETERMINED AMOUNTS OF HYDROXYETHYLVALINE (HOEtVal) IN GLOBIN SAMPLES AS A FUNCTION OF ADDED AMOUNTS OF HYDROXYETHYLATED AND [$^2H_4$]HYDROXYETHYLATED GLOBIN[a]

HOEtVal added (nmol/g Hb)	[$^2H_4$]HOEtVal added (nmol/g Hb)	HOEtVal determined (nmol/g Hb)	Number of samples	Number of injections (total)
0	0.2	0.236	3	5
0	1.0	0.227	3	5
0	5.0	0.203	1	1
0	25.0	0.258	1	2
1.44	1.0	1.56[b]	2	4
7.2	1.0	7.34[b]	3	5
36.0	1.0	36.0[b]	2	5
36.0	25.0	35.7[b]	1	2
180	1.0	154[b]	3	4

[a] Samples were produced by adding 4–500 μg ethylene oxide-alkylated globin (18 nmol HOEtVal/mg) and 4–500 μg [$^2H_4$]ethylene oxide-alkylated globin (2.5 nmol [$^2H_4$]HOEtVal/mg) to 50 mg untreated globin.

[b] Not corrected for background level of HOEtVal in the untreated globin.

amounts of alkylvalines under the present conditions of analysis.

The analytical error, expressed as the coefficient of variation, of individual GC/MS determinations was estimated from the data in Table 2 to about 6% at mean values 0.2–7 nmol/g Hb. This uncertainty is almost exclusively due to variation between injections of the same sample, probably due to operator and instrumental factors affecting peak integration. The contribution to the error from derivatization is practically nil. At higher contents (36 nmol/g Hb) the analytical error is smaller, about 1%. The contents of HOEtVal and [$^2H_4$]HOEtVal in the respective globins alkylated *in vitro* were quantified by the amino acid analyzer. The analytical error of these determinations may have introduced a systematic error of up to 10% in the GC/MS determinations.

Sensitivity. In its present form the detection limit of N-terminal alkylvalines, as exemplified by hydroxyethylvaline, is on the order of magnitude of 0.01 nmol/g globin in human samples, which corresponds to about 1 pg of injected valine. Differences in detection limits between different alkylvalines and between samples from different species are seen and are due to the variations in disturbing peaks close to those of the alkylvalines to be determined. The given detection limit should be seen as a mean value for several alkylvalines under practical conditions.

The same factors which were found to improve reproducibility also increased sensitivity above the level reached initially (17); these factors comprise changed conditions for derivatization (lower levels of by-products), purification of the extract, and introduction of on-column injection (approximately 10-fold improvement over the split/splitless injector). It was repeatedly observed that previous use of the column for other purposes could lead to considerable decrease of sensitivity, probably due to contaminants catalyzing the decay of PFPTHs. This indicates that for the maintenance of good analytical conditions one and the same column should be used exclusively for the determination of alkylvaline PFPTHs.

Background hydroxyethylvaline levels in human and rodent hemoglobins. In hemoglobin samples from humans and animals without known exposure, background levels of simple alkyl adducts such as methyl- (28) and hydroxyethyl (29–31) have been found. The sources of these alkylations—at present under investigation—may be both exogenous and endogenous (cf. Table 1). In the present study samples from 43 human males and a few

TABLE 3

OCCURRENCE OF HYDROXYETHYL ADDUCTS IN HEMOGLOBIN (Hb) FROM HUMANS AND ANIMALS WITHOUT KNOWN EXPOSURE TO HYDROXYETHYLATING AGENTS (nMOL/G Hb)

Species	Adduct to	Range	Number of samples	Ref.
Man	His^r	<0.02–4.7	31	(29)
Man	His^r	0.17–1.5	17	(30)
Man	Val	0.12–0.72	15	Present study
		0.03–0.8	43[a]	Present study[a]
Rat	His^r	1.3, 2.8	2[b]	(31)
Rat	Val	ca. 0.1	3[b]	Present study
Mouse	Val	0.02–0.12	5[b]	Present study
Syrian golden hamster	Val	ca. 0.1	4[b]	Present study

[a] Includes 28 additional samples determined with a somewhat changed method (Törnqvist and Kautiainen, to be published).

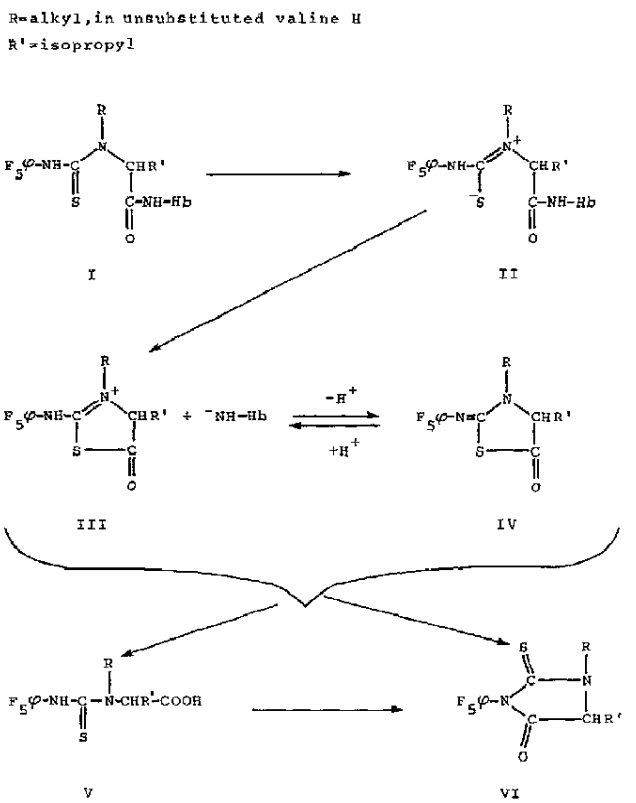
[b] Pooled samples.

mouse, rat and hamster samples were examined with regard to hydroxyethyl adducts. The found values are compatible with or somewhat lower than levels of histidine adducts found in earlier studies; see Table 3. In human and mouse hemoglobin, His-N^{τ} and terminal valine-NH_2 show approximately the same reactivities, per unit amount of globin, toward ethylene oxide (20,32,41). It is of interest to note that the human samples exhibit a very broad variation around the median value, ca. 0.3 nmol HOEtVal/g Hb.

Quantification of the lowest levels in mouse samples was estimated to have an uncertainty of a factor of 2. This is partly due to a disturbing peak, which is much higher in rodents than in humans, appearing close to the HOEtVal-PFPTH peak.

DISCUSSION

Mechanism. Some knowledge of the mechanism is important for an understanding of difficulties and ways to overcome them. In the classical Edman method for protein sequence determination (15) the phenylthiocarbamoyl derivative (corresponding to I, with R = H, in Formula 1) of the protein formed in alkaline solution is cleaved in strongly acid solution, giving rise to an anilinothiazolinone (**III**). The endpoint of this reaction is described as an equilibrium rather than a quantitative reaction (33). It is reasonable to assume that if valine is alkylated, the alkyl (R in Formula 1), through its electron-donating property, leads to an increase of the negative charge on the sulfur (**II**), thus facilitating ring closure (**III**) and cleavage of the bond to the peptide resi-



FORMULA 1

due. (This rearrangement may also proceed through other mechanisms, partly operating simultaneously.) The formed thiazolinone cation (III) may lose its charge through deprotonation of the anilino nitrogen (IV). This might, at least formally, be considered an explanation of the cleaving off of the alkylated but not of unsubstituted valines at neutral pH.

Thiazolinones (III, IV) are then converted to PFPTHs (VI). There are two possible pathways for this conversion: rearrangement to PFPTH or hydrolysis to PTC-amino acid (V) followed by ring closure to the PFPTH. The exact course of this reaction is unknown. With unalkylated amino acids (R = H) the reaction V → VI is enhanced by low pH (34). It is supposed that both pathways operate and the rearrangement may be predominating under the nearly nonhydrolytic conditions (35) in the medium, viz., formamide with a small percentage water, applied in the present derivatization procedure.

It should be considered whether the OH group of hydroxyalkylvalines plays a role in the formation of the alkylvaline PFPTH derivative. The demonstration that hemoglobin-bound methylvaline is also determined by the same method (see Fig. 3) shows that this possibility does not apply.

As the endpoint of the normal Edman degradation reaction is described as an equilibrium (33), a small amount of nonalkylated N-terminal valine of globin could also be expected to be cleaved off simultaneously with substituted valine during derivatization. In a globin sample where 1 out of 10^3 N-terminal valines was alkylated, alkyl-valines and valine peaks were of approximately equal size (see Fig. 3). This contribution from unalkylated valine does not disturb identification and determination of any of the investigated alkylated valines.

Sample derivatization and work-up procedures. Many different reaction conditions with variation of solvent, pH, amount of reagent, temperature, time, and solvent for extraction have been tried during the development of the method. The optimization of the yield of PFPTHs was complicated by the conflicting requirements regarding solvent (polar and not too lipophilic for the globin and lipophilic for the reagent) as well as the pH (optimum yield is in the pH range where globin is apt to precipitate).

Another problem concerned the formation of by-products, which is also influenced by the above-mentioned factors. One important side reaction is the hydrolysis of pentafluorophenyl isothiocyanate (PFPITC) to pentafluoroaniline followed by the formation of bis-(pentafluorophenyl)thiourea [cf. Ref. 15)]. The system for derivatization described by Mowrer *et al.* (17) is advantageous except for the formation of by-products through reaction of PFPITC with the solvent, 1-propanol. In the modified Edman degradation procedure, the steps of the formation of alkylvaline PFPTHs [cf. Ref. (15)] are not separable and the PFPTHs are therefore coextracted with large amounts of undesired by-products. To make analysis of picomole quantities possible purification of the derivatization extracts had to be introduced. In efforts to find a simple purification method, washing the extract with an alkaline water solution was tried. This leads to hydrolysis of impurities as well as of alkylvaline PFPTHs, but hydrolytic conditions permitting selective hydrolysis of many impurities could be identified. In the search for a reaction medium that gives lesser amounts of by-products, and which was suitable with regard to such selective hydrolysis, it was found that formamide could fulfill most of the requirements discussed. A purification procedure which works well combined with derivatization in formamide was developed, based on washing in a two-phase system which takes into consideration base strength of the water phase as well as polarity and volatility of the organic solvent and of the PFPTHs. This procedure for derivatization and purification has decisively increased the analytical sensitivity under practical conditions, the purity being improved by several orders of magnitude. The purification procedure is also fast and, since

it does not require fractionation of samples, it permits determination of several hemoglobin adducts (as alkylvaline PFPTHs) in the same sample.

Certain details of the procedure, such as purification of reagent [to diminish formation of bis(pentafluorophenyl)thiourea, which often interferes with determination of HOEtVal-PFPTH] and influence of pH of reaction, will be reported in a forthcoming publication. It should, however, be mentioned here that heptane and probably other alkanes are not suitable solvents for standard PFPTHs and should not be used for the extraction of PFPTH from formamide.

Sensitivity. The practical detection level, ca. 0.01 nmol HOEtVal/g Hb in human samples, corresponds to a degree of alkylation obtained from exposure to ethylene oxide at an average level of 0.01 ppm 40 h/week during more than 4 months, the life span of hemoglobin in man. This level is 500 times less than that which could be obtained from exposure at the Swedish TLV, 5 ppm. It appears that the method is sensitive enough to permit determination of tissue doses not only in work environments but also in the general environment. In the case of ethylene oxide or alkylating agents with similar reaction patterns (5), the level 0.01 nmol *N*-alkylvaline/g Hb caused by chronic or intermittent exposure, is estimated to be associated with a cancer risk around 10^{-5} per year (13,25,36). This means that already in its present state the method permits monitoring at levels down to the range where risks are becoming acceptably small (37). (The given detection level, 0.01 nmol/g Hb, corresponds to one alkylvaline per about 5×10^6 N-terminal valines or globin chains. Due to the relatively rapid repair of DNA damage this level of valine adducts of compounds with a reaction pattern similar to that of ethylene oxide (32) is estimated during chronical exposure to coexist with appreciably less than one guanine-O^6 adduct per genome.)

A further increase of sensitivity is, however, still desirable (38) and has been shown to be possible through optimization of the derivatization reaction toward higher yield (present procedure 35–40%) and further purification of reagents and solvents and of globin samples before and after derivatization. Alternative regents and radioactive labeling (18) may also be considered for this purpose.

Feasibility. The determination of amino acid adducts in total hydrolysates of protein (see Introduction) required a very time-consuming isolation of pertinent fractions containing the alkylhistidines, etc., to be determined. As compared to that procedure the determination of substituted valines using the present method (which can be made even more effective, especially with regard to isolation of globin) implies a gain in time and, consequently, costs by a factor of 10–100. One advantage is that one and the same sample can be used for the simultaneous determination of several valine adducts, whereas in the work with hydrolysates each adduct to be determined has to be subjected to fractionation–isolation and derivatization procedures.

It appears that the monitoring of environmental pollutants by N-terminal protein adducts offers an advantage of a specific kind, viz., absence of interference of erroneous incorporation of alkylated amino acids in protein synthesis *in vivo*. Such incorporation of amino acids occurs with chemically changed residues in the interior of globin chains (39).

Types of adducts determined. In its present form the method is suited for the identification and determination of low-molecular-weight alkyl adducts as has been demonstrated with methyl, hydroxyethyl, hydroxypropyl and hydroxyphenylethyl adducts. Since the method is relatively mild it permits the determination of several adducts which are unstable under the conditions of protein hydrolysis. In principle, also a priori unknown adducts could be identified and determined. Preliminary studies show that heavier adducts, such as derivatives of PAH-epoxides, are derivatized and cleaved off under the reaction conditions (Osterman-Golkar, unpublished work). For such compounds modifications of the present method may have to be developed with respect to iso-

thiocyanate reagent and procedures for separation and detection.

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